American Journal of Medical Genetics (Neuropsychiatric Genetics) 67:401–405 (1996)

Missense Mutation of the Cholecystokinin B Receptor Gene: Lack of Association With Panic Disorder

Tadafumi Kato, Zhe Wu Wang, Tomas Zoega, and Raymond R. Crowe

Department of Psychiatry, University of Iowa College of Medicine, Iowa City, Iowa (T.K., Z.W.W., R.R.C.); National University Hospital, Reykjavik, Iceland (T.Z.)

Cholecystokinin tetrapeptide (CCK_4) is known to induce panic attacks in patients with panic disorder at a lower dose than in normal controls. Therefore, the cholecystokinin B (CCK_B) receptor gene is a candidate gene for panic disorder. We searched for mutations in the CCK_B gene in 22 probands of panic disorder pedigrees, using single-strand conformation polymorphism (SSCP) analysis. Two polymorphisms were detected. A polymorphism in an intron (2491 C → A) between exons 4 and 5 was observed in 10 of 22 probands. A missense mutation in the extracellular loop of exon 2 (1550 G → A, Val^{125} → Ile) was found in only one proband. This mutation was also examined in additional 34 unrelated patients with panic disorder and 112 controls. The prevalence rate of this mutation was 8.8% in patients with panic disorder (3/34) and 4.4% in controls (5/112). The mutation did not segregate with panic disorder in two families where this could be tested. These results suggest no pathophysiological significance of this mutation in panic disorder.

KEY WORDS: anxiety disorder, single-strand conformation polymorphism, molecular genetics

INTRODUCTION

Panic disorder is characterized by recurrent panic attacks. Panic attacks can be pharmacologically precipitated. Sodium lactate [Pitts and McClure, 1967], carbon dioxide [Gorman et al., 1984], caffeine [Charney et al., 1985], and cholecystokinin tetrapeptide (CCK_4) [Bradwejn et al., 1990; de Montigny, 1989] are capable of provoking panic attacks in patients with panic disorder. CCK_4 is of particular interest because it is a specific agonist of the cholecystokinin-B (CCK_B) receptor and may play a role in the regulation of anxiety [Crawley and Corwin, 1994]. CCK_4 precipitates panic attacks in both normals and patients with panic disorder, and the nature of the attacks is similar, although a lower dosage is required in panic-disorder patients [Bradwejn et al., 1991]. Thus, patients with panic disorder appear to be more sensitive to CCK_4 than normals.

The CCK receptor exists in two forms: CCK_A and CCK_B. The CCK_B receptor is more abundant in the brain and has greater affinity for CCK_4 [Wank et al., 1994]. Therefore, a mutation in the gene that codes for the CCK_B receptor could theoretically increase the affinity of the receptor for CCK and predispose to panic disorder.

Panic disorder shows substantial familial aggregation, although it does not follow a classical Mendelian inheritance pattern [Crowe et al., 1983; Woodman and Crowe, 1995]. The familial aggregation makes genetic linkage an attractive strategy for searching for disease genes [Crowe, 1994; Wang et al., 1992]. Recently, we found a modest positive lod score between a polymorphic marker at the CCK_B receptor locus (CCKRB) and panic disorder [Wang et al., in preparation]. This finding is compatible with a mutation at the CCKRB locus causing panic disorder in a small proportion of the pedigrees. Since linkage cannot detect rare mutations, we decided to search for them directly with single-strand conformation polymorphism analysis (SSCP) [Orita et al., 1989]. The CCKRB gene has been sequenced and has five exons located at chromosome 11p15.4. The gene makes two mRNA variants by alternative splicing, referred to as "long form" and "short form" [Pisegna et al., 1992; Lee et al., 1993; Song et al., 1993]. In this study, coding regions and an alternative splicing site of the CCK_B receptor gene were examined by SSCP.

MATERIALS AND METHODS

Subjects

Subjects comprised 22 patients (13 women and 9 men, mean age ± SD of 41.8 ± 8.2 years) with DSM-III-R

Received for publication October 19, 1995; revision received February 21, 1996.

Address reprint requests to Raymond R. Crowe, M.D., Department of Psychiatry, Medical Education Building, University of Iowa College of Medicine, Iowa City, IA 52242-1000.

[American Psychiatric Association, 1987] panic disorder, with or without agoraphobia. They were probands of the pedigrees in the Iowa linkage study of panic disorder [Crowe, 1994]. Ascertainment criteria required a proband with these diagnoses and a minimum of 3 additional relatives affected with panic attacks occurring over two or more generations. Families with a history of bilineal transmission were excluded. Recruitment was from the Psychiatry Clinic of the University of Iowa Hospitals and Clinics as well as referral from other clinics in the Midwest of the USA.

Patients were assessed by psychiatrists or senior psychiatry residents using the Schedule for Affective Disorders and Schizophrenia (SADS-L) [Spitzer and Endicott, 1979] or the Structured Clinical Interview for DSM-III-R (SCID-R) [Spitzer et al., 1987].

SSCP Analysis

All samples were examined by SSCP analysis under two different conditions: 1) PCR was performed incorporating ^{35}S-dATP, and run at room temperature in a 6% polyacrylamide gel containing 5% glycerol. 2) PCR product without radioisotope was run at 4°C in a 6% polyacrylamide gel without glycerol. The gel under condition 1 was exposed to X-ray film, while the gel under condition 2 was stained with silver staining.

Total DNA was extracted from cultured lymphoblastoid cell lines by phenolchloroform extraction. Eighty nanograms of DNA from the subjects were amplified with a Thermal Cycler (Perkin Elmer, Foster City, CA) in an 8-ul reaction containing 2 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.15 mM MgCl, 0.2 μM of each oligonucleotide primer, 0.31 mM each of dCTP, dTTP, and dGTP, 0.078 mM dATP, 2.5 μCi ^{35}S-dATP, and 0.5 U Taq polymerase.

Under condition 2, the same amount of DNA was amplified in the same PCR reaction, except for containing 0.2 mM dNTP, and without ^{35}S-dATP. Taq polymerase was purchased from Gibco BRL and ^{35}S-dATP was purchased from Amersham Co., Ltd. (Buckinghamshire, England).

Oligonucleotide primers to amplify five exons and an alternative splicing site of the CCK_B receptor gene were synthesized in the DNA facility of the University of Iowa. These five exons have <300 base pairs except for exon 5, which has 548 base pairs. Exons 1–4 were amplified by one primer set each. Exon 5 was amplified by four primer sets (Table I). An alternative splicing site [Song et al., 1993] of exon 4 was amplified by another primer set (Ex4b-F and Ex4b-R).

The parameters of the PCR reaction were as follows: 94°C for 30 sec, 50–60°C for 30 sec, and 72°C for 30 sec. Before the first cycle, heat-denaturation was performed at 94°C for 3 min, and final extension was done at 72°C for 5 min.

Electrophoresis

In the experiments using ^{35}S, the PCR products were diluted 10-fold with water and again 5-fold with loading buffer containing 99% deionized formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol. These samples were heat-denatured at 94°C for 5 min and chilled on ice thereafter. Samples without formamide were also loaded to verify the position of double-strand DNA. These samples were loaded in 6% nondenaturing polyacrylamide gel containing 5% glycerol with a 1:49 ratio of acrylamide and bisacrylamide. Electrophoresis was performed in 0.5 × Tris borate buffer at 25 W for 3 hr. Electrophoresis was performed at room temperature with cooling by a fan. After electrophoresis, the gel was exposed to X-ray film for 4–7 days.

For silver-staining experiments, the PCR product was diluted 5-fold by the loading buffer and electrophoresed in 6% nondenaturing polyacrylamide gel without glycerol at 4°C for 3 hr. After electrophoresis, the gel was silver-stained [Bassam et al., 1991].

Sequencing

The shifted band was cut out from the silver-stained gel, and the DNA was eluted in Tris-EDTA buffer. The region of interest was amplified by the same primer set, using this solution as a template. The amplified PCR

TABLE I. Sequence of Primers*

Name	Sequence	Position
Ex1-F	5-TCGAGCTGAGTAAGGCGGC	−35−−17
Ex1-R	5-GTGAGAAATAGCTTGTGGGG	192–173
Ex2-F	5-ACCTCTCCCTTTTCTTACCCAG	1307–1328
Ex2-R	5-GGTGGGTGGTTGTCTCACCCA	1602–1582
Ex3-F	5-CCTTCTCTTCCCTTGTTTAG	1725–1744
Ex3-R	5-TAGTTTATGGGCAAGCTCAC	2014–1995
Ex4a-F	5-TCTGTCTGTGTTGCCTTCAGGTC	2282–2304
Ex4a-R	5-TCGCCCAGATTTTGCTCACG	2474–2493
Ex4b-F	5-GTGGCCTACGGGCTTATCTCTCGCGAGCTCTACTTA	2354–2389
Ex4b-R	5-TCCCAGCTCAACTCCATTTC	2589–2570
Ex5a-F	5-CGCCTTTTTCTCTGACCGCCCACCCTTTGTGCTCAG	2631–2667
Ex5a-R	5-TTGGCACTATAAACTGGCAACC	2913–2892
Ex5b-F	5-ATGTTGCTGGTGATCGTTGTGC	2855–2876
Ex5b-R	5-ACGGTGCATGAAGCAGTAGACC	3040–3019
Ex5c-F	5-CCTATCTCCTTCATTCACTTGC	2966–2987
Ex5c-R	5-AGCCTGGACAGCGAAGCAAT	3163–3144
Ex5d-F	5-TTCATGCACCGTCGCTTTCG	3029–3048
Ex5d-R	5-TGTCATTTGCCCTGCCTCAACC	3240–3219

*Numbers in position column represent nucleotide numbers by Song et al. [1993].

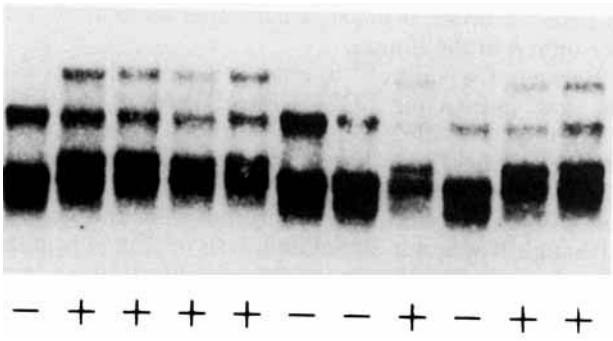

Fig. 1. SSCP analysis of exon 2 in the CCK-B receptor gene in members of the family carrying the mutation. Primers used were Ex2-F and Ex2-R. PCR was performed incorporating ^{35}S-ATP, and the samples were electrophoresed in 6% acrylamide gel with glycerol at room temperature and exposed to X-ray film. +, lane with mutated bands; −, lane with wild-type alleles.

product was purified by a Microcon concentrator (Amicon, Inc., Beverly, MA). This sample was sequenced by the cycle-sequence method, using the AmpliCycle sequencing kit (Perkin Elmer) [Innis et al., 1988] and ^{35}S-dATP. Both forward and reverse primers were used to confirm the sequence. Experimental protocols followed that supplied with the kit, except that the annealing temperature was set at 60°C.

RESULTS

SSCP Analysis and Sequencing

Single-strand conformation polymorphisms were observed in exon 2 (Fig. 1) and the alternative splicing site of exon 4. The exon 2 polymorphism was found in one of the 22 panic disorder patients. It was inherited in a Mendelian manner in the family of this patient. Sequencing revealed the polymorphism to be a point mutation, 1550 G → A (Fig. 2). This missense mutation changes valine^{125} to isoleucine in the second extracellular loop of the CCK_B receptor (nucleotide and amino acid numbers correspond to those in Song et al. [1993]).

The polymorphism in the alternative splicing site of exon 4 was observed in 10 of 22 panic disorder patients. Mendelian transmission was confirmed in four two- and three-generation families. The fragment was sequenced and the polymorphism was found to be due to a point mutation in the intron sequence between exons 4 and 5 (2491 C → A).

Association Study

To search for a pathophysiological role of the exon 2 missense mutation in panic disorder, an association study was carried out in unrelated panic-disorder patients and controls. They were genotyped by SSCP and sequencing. The panic-disorder patients were an additional 34 unrelated panic-disorder patients (19 women and 15 men) from Iowa. The controls consisted of 26 unaffected persons (12 women and 14 men) who married into the panic-disorder pedigrees. These individuals were interviewed and determined not to have panic disorder. In addition, 86 unrelated controls (39 women and 47 men) from Iowa were also genotyped. These were parents of normal control children recruited from a birth registry in the Midwest for a study of cleft palate. They were not interviewed, and their panic disorder status is unknown.

Three (2 women and 1 man) of 34 (8.8%) additional unrelated patients with panic disorder from Iowa had this mutation. On the other hand, none of 26 unaffected subjects who married into these panic pedigrees had this mutation. Five (all women) of 86 unrelated population controls had this mutation. In total, 5 of 112 (4.4%) unrelated controls had this mutation. The

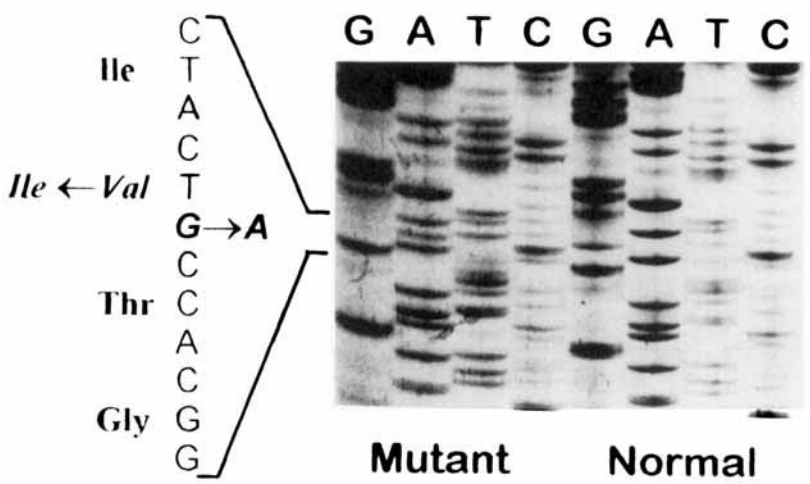

Fig. 2. Sequence ladders of exon 2 in the CCK-B receptor gene. Left ladder shows a mutated gene. Right ladder shows a wild-type gene. Point mutation 1550 G → A, which causes an amino acid change from valine^{125} to isoleucine, is seen.

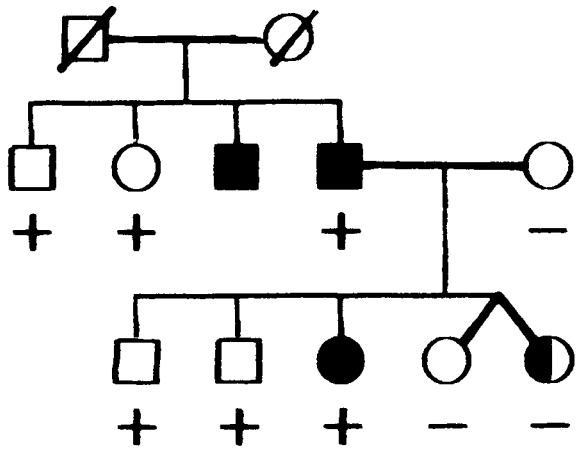

Fig. 3. A pedigree of the family with the mutation 1550 G → A in Iowa. Solid circle or square indicates a person affected with panic disorder, and a half solid circle indicates possible panic disorder. +, subject with a mutated allele; −, subject with no mutated alleles.

difference in prevalence rates of this mutation between panic patients and controls in midwestern Americans (8.8% vs. 4.4%) was not statistically significant. Therefore, the mutation was not associated with panic disorder.

Segregation of the Polymorphism and Panic Disorder

Five panic disorder probands (4 women and 1 man) from multiplex pedigrees ascertained in Iceland were also examined. One of 5 probands of the Icelandic families had this mutation. All family members of these two probands of multiplex pedigrees (Iowa and Iceland) were genotyped. The results show no evidence of cosegregation between the mutation and panic disorder (Figs. 3, 4).

DISCUSSION

To our knowledge, this is the first evidence of a missense mutation in a candidate gene for panic disorder. However, the mutation does not play an etiological role in panic disorder, because it is neither associated with nor linked to the illness.

Because the valine[125] in exon 2 is conserved in dog, rat, and guinea pig CCK_B genes [Wank et al., 1994; Pisegna et al., 1992; Lee et al., 1993], substitution of this amino acid may have functional significance. Because this amino acid is in an extracellular loop, it could affect the affinity of this receptor for its agonists. Although it was not associated with or linked to panic disorder, we cannot rule out the possibility that it may be a risk factor for other neuropsychiatric disorders. CCK regulates not only anxiety but also appetite, respiratory function, sleep, and seizure threshold [Crawley and Corwin, 1994]. It also interacts with dopamine [Crawley and Corwin, 1994]. Therefore, it may be worthwhile to search for association or linkage of this mutation to other psychiatric disorders, such as schizophrenia and eating disorders.

It should be noted that the number of patients examined in this association study was not large enough to exclude possible association. Assuming a control rate of 0.05 and a patient rate of 0.10, the probability of detecting a significant difference at the 0.05 probability level (two-sided) is 0.16 in this sample size.

The sensitivity of mutational detection by SSCP is not 100%. The DNA fragments examined by SSCP in this study were 185–295 bp (average, 236 bp). If we assume that the sensitivity of mutational detection of SSCP in this size of DNA fragment is 80%, 96% of mutations can be detected when they are examined under two different experimental conditions. Therefore, we can assume that the sensitivity of mutational detection in this study is close to 100%.

ACKNOWLEDGMENTS

We are grateful to Dr. Andrew C. Lidral and Dr. Jeffrey C. Murray (Department of Pediatrics, University of Iowa College of Medicine) for the DNA samples of normal controls. This study was supported by grants from the National Institutes of Health (MH-34728 and MH-00735) to R.R.C.

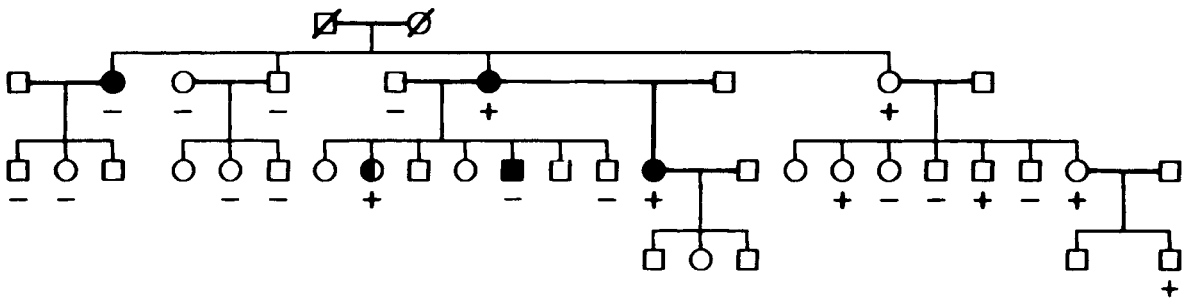

Fig. 4. A pedigree of the family with the mutation 1550 G → A in Iceland. Symbols are the same as those in Figure 3.

REFERENCES

American Psychiatric Association (1987): " Diagnostic and Statistical Manual of Mental Disorders, 3rd Edition, Revised." Washington, DC: American Psychiatric Press.

Bassam BJ, Caetano-Anolles G, Gresshoff PM (1991): Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem 196:80–83.

Bradwejn J, Koszycki D, Meterissian G (1990): Cholecystokinin-tetrapeptide induced panic attacks in patients with panic disorder. Can J Psychiatry 35:83–85.

Bradwejn J, Kosztcki D, Shriqui C (1991): Enhanced sensitivity of cholecystokinin-tetrapeptide in panic disorder: Clinical and behavioral findings. Arch Gen Psychiatry 48:603–607.

Charney DS, Heninger GR, Jatlow PI (1985): Increased anxiogenic effects of caffeine in panic disorder. Arch Gen Psychiatry 42: 233–243.

Crawley JN, Corwin RL (1994): Biological actions of cholecystokinin. Peptides 15:731–755.

Crowe RR (1994): The Iowa linkage study of panic disorder. In Gershon ES, Cloninger CR (eds): "Genetic Approaches to Mental Disorders." Washington, DC: American Psychiatric Press, pp 253–272.

Crowe RR, Noyes R, Pauls DL, Slymen D (1983): A family study of panic disorder. Arch Gen Psychiatry 40:1065–1069.

De Montigny C (1989): Cholecystokinin tetrapeptide induces panic-like attacks in healthy volunteers. Arch Gen Psychiatry 46: 511–517.

Gorman JM, Askanazi J, Liebowitz MR, Flyer AJ, Stein J, Kinney J, Klein DF (1984): Response to hyperventilation in a group of patients with panic disorder. Am J Psychiatry 141:857–861.

Innis MA, Myambo KB, Gelfand DH, Brow MAD (1988): DNA sequencing with Thermus aquatics DNA polymerase and direct sequence determination. Proc Natl Acad Sci USA 85:9436–9440.

Lee YM, Beinborn M, McBride EW, Lu M, Kolakowski LF Jr, Kopin AS (1993): The human brain cholecystokinin-B/gastrin receptor. Cloning and characterization. J Biol Chem 268:8164–8169.

Orita M, Suzuki Y, Sekiya T, Hayashi K (1989): Rapid and sensitive detection of point mutation and DNA polymorphisms using the polymerase chain reaction. Genomics 5:874–879.

Pisegna JR, de Weerth A, Huppi K, Wank SA (1992): Molecular cloning of the human brain and gastric cholecystokinin receptor: Structure, functional expression and chromosomal localization. Biochem Biophys Res Commun 189:296–303.

Pitts FN, McClure JN (1967): Lactate metabolism in anxiety neurosis. N Engl J Med 277:1329–1336.

Song I, Brown DR, Wiltshire RN, Grantz I, Trent JM, Yamada T (1993): The human gastrin/cholecystokinin type B receptor gene: Alternative splicing donor site in exon 4 generates two variant mRNAs. Proc Natl Acad Sci 90:8095–9089.

Spitzer RL, Endicott J (1979): "Schedule for Affective Disorders and Schizophrenia (Lifetime Version), 3rd Edition." New York State Psychiatric Institute, Biometrics Research.

Spitzer RL, Williams JBW, Gibbon M (1987): "Structured Clinical Interview for DSM-III-R." New York: New York State Psychiatric Institute, Biometrics Research.

Wang ZW, Crowe RR, Noyes R (1992): Adrenergic receptor genes as candidate genes for panic disorder: A preliminary study. Am J Psychiatry 149:470–474.

Wank SA, Pisegna JR, de Weerth A (1994): Cholecystokinin receptor family. Molecular cloning, structure, and functional expression in rat, guinea pig, and human. Ann NY Acad Sci 713:49–66.

Woodman C, Crowe RR (1995): The genetics of panic disorder. In Asnis GM, von Praag HM (eds): "Panic Disorder. Clinical, Biological, and Treatment Aspects." New York: John Wiley & Sons, pp 66–79.